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Vegetative λ DNA

III.† Pulse-labeled Components

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The structures of λ DNA labeled during short pulses of [^{3}H]thymine at various times after infection have been studied by ultracentrifugal analysis. Circular DNA's, both the supercoiled form and an open form, are the main DNA components synthesized in the first half of the latent period. The open circular form is the most rapidly synthesized and may be a precursor to the supercoiled DNA. During the later period of progeny DNA accumulation the open circular form and a heterogeneous, more rapidly sedimenting component(s) are synthesized during such pulses. These are apparently involved in the generation of the linear, viral DNA.

1. Introduction

After infection of a sensitive or immune bacterium with bacteriophage λ, a large fraction of the viral DNA is converted to two fast-sedimenting forms (Young & Sinsheimer, 1964; Bode & Kaiser, 1965). Component I or RF I‡ is a closed-circular, "supercoiled" form of λ DNA analogous in structure to φX RF I or polyoma viral DNA component I. Component II is an open-circular form which contains at least one phosphodiester scission and, again, is analogous to the corresponding φX or polyoma form (Bode & Kaiser, 1965; Young & Sinsheimer, 1967*b*; Ogawa & Tomizawa, 1967). The circular DNA forms are able to infect spheroplasts but not helper-infected cells (Young & Sinsheimer, 1967*a*).

λ RF accumulates specifically in the early part of the latent period, prior to the synthesis of any linear λ DNA. Concomitant with the appearance of intracellular phage, net synthesis of the RF ceases and synthesis of helper-infectious, linear phage DNA begins (Young & Sinsheimer, 1967*b*). The conversion to, and synthesis of RF explain the eclipse of λ DNA infectivity observed in the helper assay by Dove & Weigle (1965).

The experiments to be described were performed in an attempt to identify the precursors to both RF and viral DNA, and to study in a preliminary way the structure of "replicative intermediates." The rationale of the experiments was to label λ-infected bacteria for periods of time short enough that a significant fraction of the labeled molecules would be only partially replicated. With longer exposures to label, more

† Paper II in this series is Young & Sinsheimer (1967*b*).

‡ Hereafter the circular vegetative λ DNA will be called λ RF or simply RF if there is no possibility of confusion with φX RF. As in φX, RF I refers to the closed-circular (supercoiled) form and RF II to the open-circular form. Unless otherwise stated, λ RF means RF I.

completed molecules will be present in the labeled population and some relation between incomplete (i.e. precursors) and completed molecules should become evident.

The results confirm the general outline of λ DNA replication presented previously (Young & Sinsheimer, 1967*b*). RF is synthesized almost exclusively at early times in the latent period and phage DNA at late times. These results, together with the earlier data, indicate that RF I is not a major material precursor to phage DNA. However, RF II is synthesized both early and late, suggesting that it may be involved in the replication of both RF I and phage DNA. A heterogeneous component(s) sedimenting faster than RF II also seems to be a precursor to phage DNA.

2. Procedures

The techniques and materials have been described in previous papers (Young & Sinsheimer, 1967*a*,*b*).

(a) *Outline of a typical pulse experiment*

In all the experiments presented in the Results section, *Escherichia coli* AB2500 (a multiple auxotroph which is T^- (thymine-requiring) and *uvrA-6* (defective in DNA repair), the two important loci for the present purposes (Howard-Flanders, Boyce & Theriot, 1966) was infected with λ *c26* at a multiplicity of 2 to 5 plaque-forming units/cell at 37°C in TM buffer for 5 min and then diluted into warmed (37°C) KG medium containing 5 μg/ml. thymine. The bacteria are pre-treated with 25 μg equivalents/ml. of mitomycin C to inhibit host DNA synthesis (Lindqvist & Sinsheimer, 1966; Young & Sinsheimer, 1967*b*). About 5 min before adding radioactive thymine for the pulse, uracil is added at a final concentration of 5 μg/ml. to suppress uptake of label into RNA. At the desired time [^{3}H]thymine (Schwarz Bioresearch, Inc., Orangeburg, N.Y.) is added. Incorporation is stopped by pipetting the culture into a heavy-wall glass centrifuge tube in a dry ice–acetone bath. The tube contains an excess of unlabeled thymine. At the end of the experiment the frozen cultures are thawed at 5°C and the infected bacteria are gently pelleted by 4 min centrifugation at 2500 *g*. The pellet is resuspended in sucrose and the DNA extracted as described previously. We find that attempts to collect and wash the cells by filtration usually result in losses of 50 to 90% of the infected bacteria. Near the end of the latent period, λ-infected centers become very sticky and difficult to resuspend from filter paper or a centrifuge tube, probably because of lysis of some of the cells. The low recoveries of ^{3}H after collecting "late" cells (Table 1) are a consequence in part of cell lysis upon pelleting and in part of incomplete sedimentation of cells because of the low centrifugal force employed in an effort to minimize lysis.

3. Results

(a) *Recovery of pulse-labeled DNA from infected cells*

Since there have been prior reports of difficulty in the recovery of pulse-labeled DNA from infected (Frankel, 1966; Smith & Burton, 1966) or even uninfected (Hanawalt & Ray, 1964) bacteria, the results of the following experiment will be described in some detail with regard to the recovery.

Cultures infected with λ (as described in Procedures) were pulse-labeled with [^{3}H]-thymine for 60, 120 and 300 seconds starting at 10 and 35 minutes after infection at 37°C. The pulses starting at 10 minutes will be termed *early*, those starting at 35 minutes, *late*. From previous experiments these times correspond, respectively, to a period of synthesis of RF and phage DNA.

Table 1 indicates the radioactivity incorporated in each pulse by 5-ml. portions of the culture and compares the number of phage DNA equivalents synthesized (based on the specific activity) with the number of active phage produced measured

TABLE 1

Recovery of pulse-labeled DNA and the rate of synthesis of DNA and active phage

Time	Early (10 min)			Late (35 min)		
Pulse length (sec)	60	120	300	60	120	300
Total [^{3}H]thymine incorporation (cts/min)	54000	159000	540000	292000	500000	1070000
Recovery of [^{3}H]DNA after collecting cells (%)	65	57	71	34	41	48
Recovery of [^{3}H]DNA after phenol extraction (%) (a)	50	45	43	59	51	41
(b)	—	—	—	78	66	71
λ DNA equivalents/IC	1·8	5·3	18	9·5	16	35
Active phage/IC	—	—	—	1·8	3·6	9

The total cts/min incorporated is per 5 ml. culture. The [^{3}H]thymine specific activity in the culture was 2·6 c/m-mole and the number of DNA equivalents is based on this figure, the thymine content of λ DNA, and the known counting efficiency. At this specific activity there is about 2×10^{-4} disintegration/min/λ DNA or approximately 0·3 disintegration/day/λ DNA. The total incorporation was measured by trichloroacetic acid precipitation of a portion of the culture immediately after the pulse. The recoveries after phenol extraction are based on the amount recovered after collection of the cells. The recovery was enhanced in (b) by the addition of sodium trichloroacetate to the lysate (final concentration 0·1 M, pH 8·0) prior to phenol extraction.

from the intracellular growth curve. Intracellular phage synthesis began 25 minutes after infection. The recoveries after collection of the cells and after phenol extraction of the lysates produced by lysozyme–EDTA, sodium dodecyl sulfate, and pronase treatment (Young & Sinsheimer, 1967*b*) are also shown. The recoveries after phenol extraction in (b) in the Table are apparently enhanced by the addition of sodium trichloroacetate to the DNA solution (final concentration 0·1 M, pH 8·0) prior to phenol extraction (Weil, 1961). These lysates were extracted very gently by rolling to produce an emulsion. Even so, the recoveries are high enough to ensure that conclusions drawn from an analysis of this DNA will apply to most of the pulse-labeled DNA in the cell.

In the late pulse a large number of λ DNA equivalents are synthesized (about nine in 60 seconds). The fraction of partially replicated molecules will thus depend largely on the number of growing points per cell.

As observed previously (Young & Sinsheimer, 1967*b*), the rate of DNA synthesis is not constant at early times in the latent period but is approximately so later. Although this might imply a different mechanism of DNA replication at early and late times, such as an exponential *versus* a linear mode, it could equally well imply changes in precursor pool size or that a different component (enzyme or DNA) in the reaction is rate-limiting at early and late times, or the establishment at late times of a steady state condition of synthesis and maturation of phage genomes.

From 35 to 40 minutes the rate of DNA synthesis exceeds the rate of synthesis of active phage by about a factor of four. One might expect then to find a pool of free phage DNA in the cell, unlike the situation observed during φX infection in which no free phage is present (Sinsheimer, Starman, Nagler & Guthrie, 1962). This is also unlike the growth of T4 during which the rates of phage and DNA synthesis are nearly equal after phage maturation begins (Hershey, 1953). Weigle (personal

communication) finds that λ lysates usually contain a variable excess of full heads over tails. This might account for the "excess" DNA.

(b) *Sedimentation analysis of pulse-labeled DNA*

(i) *At low ionic strength, pH 10·5*

The distributions of the pulse-labeled [^{3}H]DNA described in Results section (a), and a sedimentation marker (^{32}P-labeled phage DNA) after centrifugation through sucrose gradients (in 10 mM-Tris–1 mM-EDTA, pH 10·5) are shown in Figures 1 and 2. The

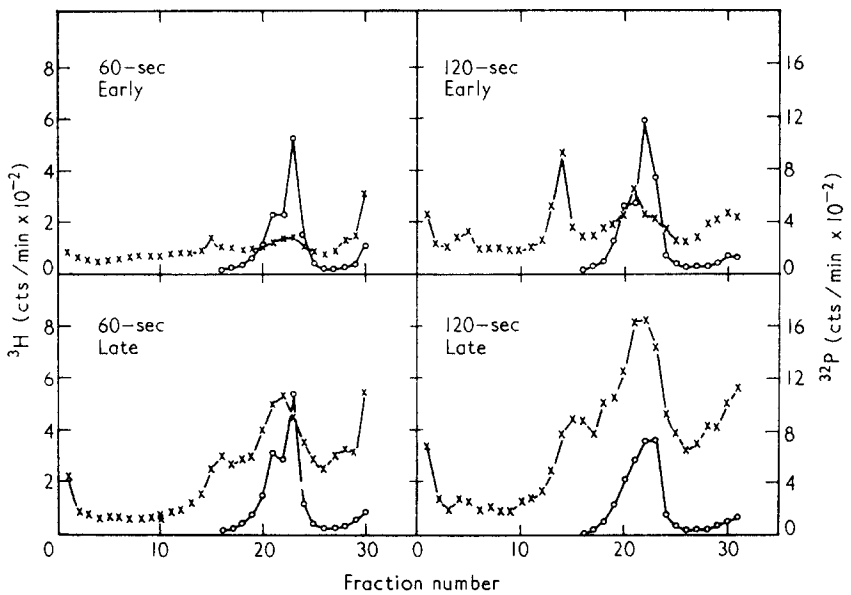

FIG. 1. Sedimentation properties of vegetative λ DNA labeled with [^{3}H]thymine for 60 and 120 sec at 10 and 35 min. 0·5-ml. portions of the lysates (phenol extracted, Table 1(a)) were mixed with ^{32}P-labeled λ *b2b5c* phage DNA and layered on 5 to 20% sucrose gradients containing TE buffer, pH 10·5, and centrifuged for 5 hr at 25,000 rev./min and 6°C in the SW25·1 rotor. 1 ml. of 55% CsCl (density = 1·7 g/cm^3) was layered on the bottom of the sucrose gradients to catch any DNA in a pellet. Sedimentation is from right to left in this and succeeding Figures. All of each fraction (about 0·85 ml.) was precipitated with trichloroacetic acid and counted in a Beckman scintillation counter. The cts/min in the ^{3}H channel were not corrected for a 2% overlap from the ^{32}P channel, causing a small amount of skewing of the slow-sedimentating peak of early, 60-sec pulsed DNA toward the top of the gradient. Recovery of ^{3}H pulse-labeled DNA layered on the gradient: 60-sec, early, 100%; 60-sec, late, 71%; 120-sec, early, 75%; 120-sec, late, 71%. —×—×—, ^{3}H cts/min; —○—○—, ^{32}P cts/min.

marker consists of a mixture of hydrogen-bonded circular and linear molecules. The fractions from the gradients containing the DNA labeled for 300 seconds (Fig. 2) were assayed for radioactivity and for infectivity in the helper and spheroplast assays. DNA infective in the spheroplast assay was measured both before and after denaturation of each fraction from the gradient. The purpose of denaturation was to reveal the sedimentation position of RF II on these gradients. Denaturation of RF II produces single-stranded rings of λ DNA, which are more infective in the spheroplast assay than native DNA; thus an increase in the infectivity after denaturaton indicates the probable presence of RF II. λ RF I has the same or slightly less infectivity after denaturation (Kiger, Young & Sinsheimer, 1967).

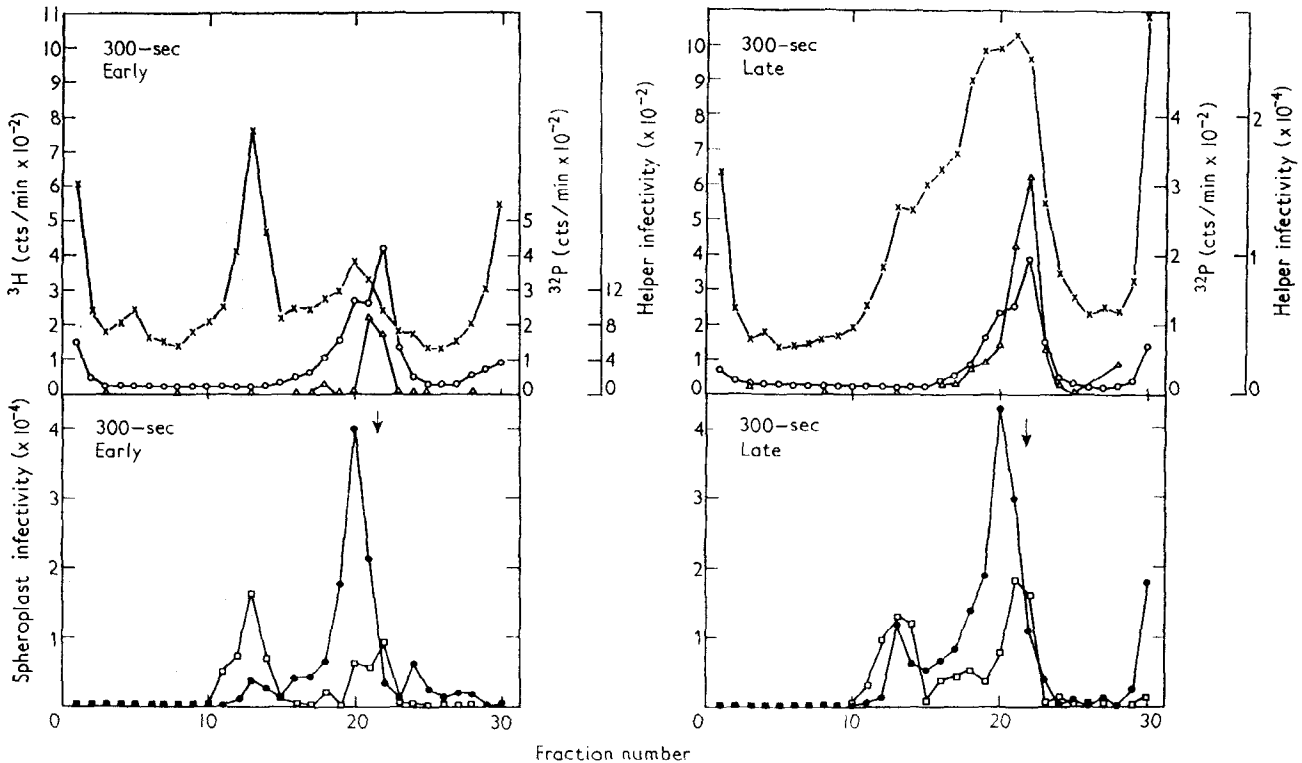

FIG. 2. Sedimentation and biological properties of the DNA extracted from 300-second pulse-labeled infected bacteria. The conditions for sedimentation are given in the legend to Fig. 1. 0·5 ml. of the phenol-extracted lysates from the infected cultures labeled for 300 sec at 10 and 35 min was centrifuged. One-half of each fraction was precipitated with trichloroacetic acid. Helper infectivity was assayed by mixing 50 μl. of either individual fractions (16 to 25) or pooled fractions (1–5, 6–10, 11–15, and 26–30), with 0·2 ml. of helper-infected cells. The infectivity in the spheroplast assay of the native DNA in each fraction was measured by adding 0·2 ml. of each fraction to 0·6 ml. of 0·05 M-Tris, pH 8·1 and mixing with 0·2 ml. of spheroplast stock. The infectivity in the spheroplast assay of the DNA after alkaline denaturation of each fraction was measured by first adding 50 μl. of 0·5 N-NaOH to 0·2 ml. of each fraction. Then, after denaturation for 15 min at 37°C, the pH was returned to about 7·5 by addition of 0·06 ml. of 0·05 M-Tris, pH 8·1 and 0·05 M-NaH_2PO_4 before mixing with the spheroplasts. The recoveries of ^{3}H layered on the gradient were: early pulse, 73%; late pulse, 63%. —×—×—, ^{3}H cts/min; —○—○—, ^{32}P cts/min; ···△···△—, helper infectivity; —□—□—, spheroplast infectivity, native; —●—●—, spheroplast infectivity, denatured.

The sedimentation properties of the early pulse-labeled DNA correspond to those of RF I and RF II. That the fast-sedimenting DNA is supercoiled and not a concatenate is substantiated in Results section (c) (*vide infra*). The slow peak of ^{3}H-pulsed DNA co-sediments with the marker H-bonded circular DNA and also with material which has the greatest infectivity increase after denaturation, indicative of the position of RF II. It is concluded that this pulse-labeled DNA is therefore RF II. The amount of RF I increases relative to RF II as the length of the pulse increases. At times near the end of the DNA helper-infectivity eclipse period (Dove & Weigle, 1965), the ratio of accumulated RF I to RF II may be five or ten to one. This suggests that RF II is a precursor to RF I.

Sedimentation of the late pulse-labeled samples reveals an entirely different distribution of components. A large fraction of the ^{3}H in the extracts from cells labeled for 60 and 120 seconds sediments as RF II. Only in the extract containing DNA labeled for 300 seconds is a peak of linear phage DNA clearly resolved (Fig. 2). In addition to these components there is a large amount of [^{3}H]DNA with a distribution of sedimentation coefficients sedimenting faster than RF II. Although some of this material sediments nearly as fast as RF I, very little behaves as supercoiled DNA when centrifuged in a CsCl–ethidium bromide equilibrium density gradient (Results section (c)).

The distribution of infectivity after centrifugation of the early and late 300-second labeled extracts reveals three things. First, the broad pattern of labeled sedimenting material in the late sample is not an artifact caused by front spreading, since the infectivity on the same gradient sediments homogeneously, without evidence of concentration dependence. (The marker also behaves normally.) Second, some of the material which sediments faster than RF II apparently releases single-stranded rings upon denaturation. This conclusion is based on the increased infectivity after denaturation which occurs in the fractions ahead of RF II. Third, there has been a 20-fold increase in the amount of helper-infective phage DNA between 15 and 40 minutes, indicating synthesis of infective phage DNA has begun and is probably occurring during the late pulse. The position of the helper-infective DNA corresponds to the position of a peak of pulse-labeled DNA on the same gradient, confirming the identity of the latter as phage DNA (Fig. 2).

(ii) *At low ionic strength, pH 10·5 and in 2·0 M-NaCl*

The sedimentation rate of RF I is very sensitive to changes in the ionic strength (Bode & Kaiser, 1965; Young & Sinsheimer, 1967*b*), having a maximum $S_{20,w}$ at about $\Gamma = 10^{-2}$ (J. Kiger, personal communication) and decreasing at higher ionic strengths. Denatured DNA is also more sensitive to changing salt concentrations than is native DNA (Studier, 1965). To investigate the effect of low and high ionic strength on the sedimentation behavior of the DNA labeled in the late 300-second pulse, portions were layered on sucrose gradients containing (a) TE (10 mM-Tris–1 mM-EDTA) pH 10·5, or (b) 2·0 M-NaCl–TE, pH 8·1 and centrifuged for six hours. Sedimentation markers, ^{32}P-labeled *b2b5c* phage DNA and purified [^{32}P]RF I, were added before centrifugation.

The results shown in Figure 3 indicate that the marker [^{32}P]RF I sediments much more slowly at high ionic strength, as expected. S(RF I)/S(λ DNA) = 2·3 at low Γ, 1·6 at high Γ. The low ionic strength gradient reveals that about 5% of the pulse-labeled DNA sediments as RF I. Peaks corresponding to RF II and phage DNA are also resolved. They are present in approximately equal proportions representing

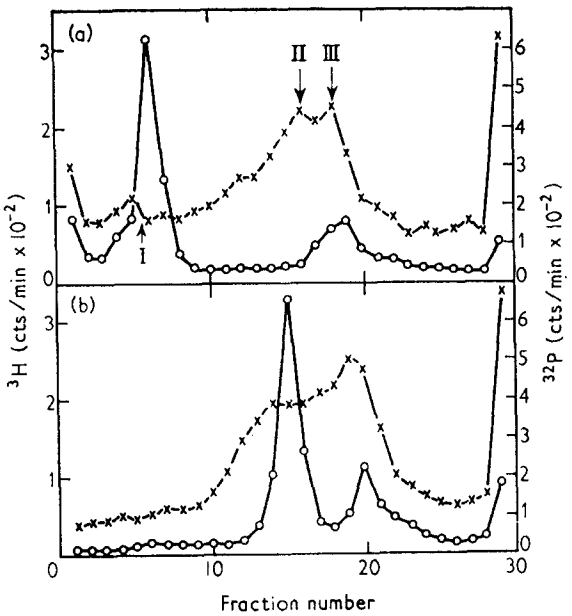

FIG. 3. Sedimentation of late 300-second pulse-labeled DNA at low and high ionic strength. Purified ^{32}P-labeled phage DNA and RF I were added as sedimentation markers prior to centrifugation for 6 hr at 25000 rev./min at about 10°C.

(a) 5 to 20% sucrose in TE (0·01 M-Tris, pH 10·5, 0·001 M-EDTA). Recovery: ^{3}H, 65%; ^{32}P, 69%.

(b) 5 to 20% sucrose in TE, pH 8·1, 2·0 M-NaCl. Recovery: ^{3}H, 63%; ^{32}P, 71%.

—×—×—, ^{3}H cts/min; —○—○—, ^{32}P cts/min.

together about 60% of the pulse-labeled DNA. Most of the remaining radioactivity on the low-salt gradient sediments heterogeneously ahead of RF II, as observed in Figures 1 and 2.

On the high-salt gradient only the peak of phage DNA is clearly resolved; the remaining radioactivity sediments heterogeneously, both faster and slower than RF I. High ionic strength minimizes the difference in sedimentation rate between phage DNA and RF II (J. Kiger, personal communication), thus RF II is not resolved on this gradient. Either the high ionic strength or the lower pH (or both) changes the sedimentation properties of a large fraction of the late, pulse-labeled DNA relative to RF I.

When the extract containing early, 300-second labeled DNA was sedimented through a sucrose gradient containing 2·0 M-NaCl, the fast peak sedimented 1·6 times as fast as phage DNA, confirming its identification as RF I.

(iii) *Without phenol extraction*

Portions of the lysates (300-second labeling period) were also sedimented after treatment with pronase and sodium dodecyl sulfate for two hours in lieu of phenol. At neutral pH the same sedimentation components just described are observed. However, somewhat more RF II is observed in the early lysate and somewhat more faster-sedimenting material is present in the late lysate. These components might be preferentially lost during phenol extraction or their structure and hence sedimentation properties may be changed by the exposure to phenol.

(c) *Identification of pulse-labeled RF I by dye binding*

Radloff, Bauer & Vinograd (1967) showed that the preferential binding of the intercalating dye, ethidium bromide, by linear or "nicked"-circular DNA as compared to supercoiled DNA could be used as a technique to separate these forms. The supercoiled DNA binds less dye and hence has a greater buoyant density in a CsCl–ethidium bromide equilibrium density gradient than linear or nicked-circular DNA. Phenol-extracted pulse-labeled DNA was mixed with ethidium bromide and CsCl and centrifuged to near equilibrium. Phage DNA, ^{32}P-labeled *b2b5c*, and purified [^{32}P]RF I were added before centrifugation.

As shown in Figure 4, about 40% of the early 300-second pulse-labeled DNA is RF I. No RF I is detectable in the late 60-second sample, but about 5% of the late 300-second pulse DNA is RF I, as can be seen by the inset to the bottom Figure. These values agree well with those obtained by sedimentation analysis.

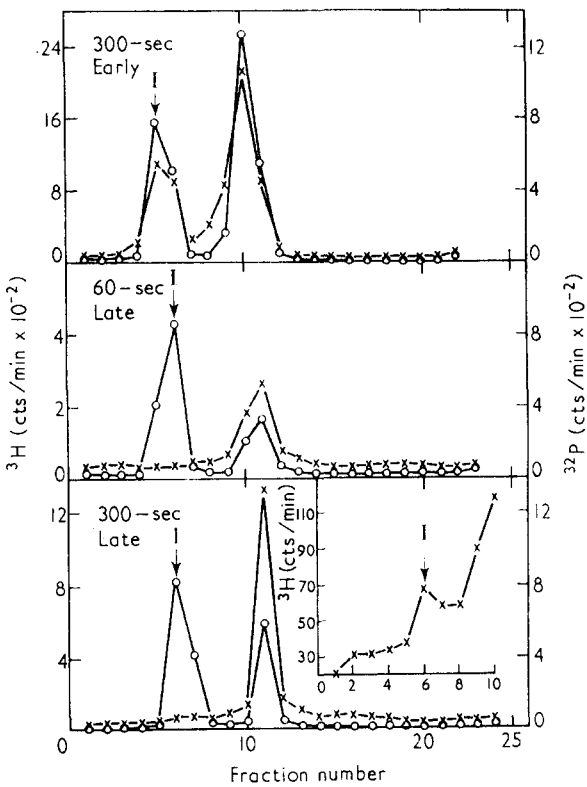

FIG. 4. CsCl–ethidium bromide equilibrium density gradient centrifugation of early and late pulse-labeled DNA. (Density increases from right to left.) Portions of the lysates to be analyzed were mixed with ^{32}P-labeled phage DNA, [^{32}P]RF I, an aqueous solution of ethidium bromide (Boots Pure Drug Co., Nottingham, England) and solid CsCl to produce a final concentration of 50 μg/ml. ethidium bromide and a density of 1·56 g/cm^{3}. The solution was centrifuged to near equilibrium (32 hr at 30,000 rev./min and 20°C). Three-drop fractions were collected, trichloroacetic acid-precipitated with carrier, and counted in the scintillation counter. The inset in the lower graph is a presentation of the data of fractions 1 to 10 on an enlarged scale.

Recoveries of radioactivity layered on the gradient: late, 60-sec, ^{3}H, 20%; ^{32}P, 79%; late, 300-sec, ^{3}H, 40%; ^{32}P, 78%; early, 300-sec, ^{3}H, 58%; ^{32}P, 80%. The cts/min in the ^{3}H channel of the scintillation counter were corrected for a 2% overlap from the ^{32}P channel. —×—×—, ^{3}H, cts/min; —○—○—, ^{32}P, cts/min.

Because the recoveries of the vegetative DNA from the gradients are so low (see legend to Fig. 4) the absence of RF I in the late 60-second pulse gradient is not proof that it was not labeled during this interval. The reason for the low recoveries is unknown; the losses are apparently not random since most of the marker [^{32}P]DNA is recovered.

(d) *Synthesis of RF I, RF II, phage DNA and component X throughout the latent period*

Synthesis of the components identified by the pulse experiment just described has been studied by pulse-labeling phage-infected bacteria for sequential 5-minute intervals from 0 to 50 minutes as described in Procedures. For comparison, one portion of the culture was labeled continuously from 0 to 50 minutes. The total radioactivity incorporated in the pulses is 80% of the amount incorporated continuously, indicating a small lag before incorporation of the isotope during each pulse. Samples of the phenol-extracted lysates were centrifuged on low-salt neutral sucrose gradients (pH 8·0) with a marker phage DNA. The gradients are divided into three regions based on the distribution of sedimentation components. Region I contains material which sediments at the rate of RF I. The relative amount of RF I in several of these samples was verified by centrifugation in alkaline sucrose. Region II includes RF II and the heterogeneous component X which sediments in the region between RF I and II. Region III includes material sedimenting in the region of linear phage DNA. The cumulative amount of radioactivity in each of these three regions is plotted in Figure 5. This plot shows the relative amounts of the various components which would be *expected* to be present in the culture labeled continuously if each component were stable and did not act as a precursor.

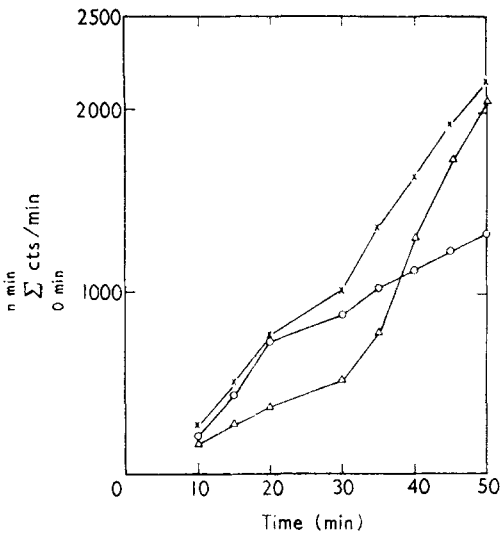

FIG. 5. Cumulative synthesis of λ-specific DNA components predicted from sequential 5-min pulses. Equal portions of lysates from 5-min pulse-labeled cultures and also from a culture labeled continuously from 0 to 50 min, were layered onto 5 to 20% sucrose gradients in TE buffer and centrifuged as usual. The amount of radioactivity in the three regions described below was measured and the cumulative sum determined at 5-min intervals. (The 0 to 5- and 20 to 25-min samples were lost.)

Region I: RF I, —○—○—,

Region II: RF II and component X (all material sedimenting between phage DNA and the RF I position), —×—×—,

Region III: phage DNA (radioactivity which co-sedimented with the phage DNA marker), —△—△—.

The results indicate that initially the rates of synthesis of RF I and RF II+X are equal (most of the latter is RF II in the early pulses). The decrease in the rate of synthesis of RF I after 20 minutes is accompanied by a very large increase in the rate of synthesis of phage DNA. The apparent early synthesis of linear phage DNA is probably an artifact as a consequence of overlap of component RF II into region III. The residual synthesis of RF I at late times is also probably more apparent (because of overlap of component X) than real, since it was shown in section (c) that very little RF I is synthesized at late times in the infection.

If all of the pulse-labeled components are stable, the ratio RF I:RF II+X: phage DNA predicted from Figure 5 is 24:39:37 at 50 minutes. (The total sum is 100%.) The ratio actually measured after sedimentation of the DNA labeled continuously from 0 to 50 minutes is 11:15:74.† One explanation for the difference between the predicted and measured values is that RF II and component X are precursors to phage DNA. RF II is probably also a precursor to RF I and this step is very likely to some extent reversible.

4. Discussion

Analyses of the DNA pulse-labeled early in the latent period confirm the conclusions reached previously (Young & Sinsheimer, 1967*b*). RF I and RF II, the closed and open-circular forms, respectively, of λ DNA are the primary components synthesized initially after infection with λ. The increasing ratio of RF I to RF II with longer periods of labeling suggests that RF II is a precursor to RF I.

The sedimentation analysis of the DNA pulse-labeled late in the latent period has little resemblance to that described by Smith & Skalka (1966). It is unlikely that this is a result of shear breakage of the concatenate since bacterial DNA extracted by the same procedure sediments uniformly at a rate twice as fast as phage DNA, the rate of Smith & Skalka's concatenate. The reason for this difference is not known.

The DNA labeled in a short pulse during the period of phage DNA synthesis sediments heterogeneously faster than mature phage DNA. Part of this DNA has the sedimentation and biological properties of RF II: it sediments with hydrogen-bonded circular DNA and with material which releases highly infective DNA upon denaturation (presumably single-stranded rings of λ DNA). This is not conclusive evidence that the pulse-labeled DNA itself contains single-stranded rings, since the infectivity represents the behavior of the bulk of the DNA whereas the pulsed DNA is only a small fraction of the total DNA on the gradients. The heterogeneous material, component X, has a range of sedimentation coefficients between (and perhaps including) those of RF I and II. At high ionic strength some of it sediments faster than RF I.

The physical nature of component X is unclear.

The sedimentation rate of the late pulse-labeled DNA is more sensitive to ionic strength than phage DNA but is less sensitive than RF I. The net effect, however, is difficult to evaluate due to the heterogeneity. High ionic strength appears to make the heterogeneous material sediment more uniformly and some of it more slowly (Fig. 3). Part of this effect may have been caused by the lower pH used on the high-salt gradients. That is, low salt (0·01 M-Tris) and moderately high pH (10·5) may have

† Because of the overlap of X with RF I in sedimentation rate, the amount of X predicted should perhaps be larger by as much as 10%—the amount of RF I synthesized after 20 min—and the amount of RF I will be correspondingly decreased. There are approximately equal amounts of RF II and X synthesized between 30 and 50 min.

caused incipient denaturation of some of the pulse-labeled DNA, thus increasing its sedimentation rate.

RF I may be synthesized in small quantities even very late (35 to 40 minutes when lysis occurs at 50 minutes) in the latent period, as indicated by the dye-binding experiments (Fig. 4). Such late synthesis of RF I is not always observed, however, and it cannot be ruled out that the late synthesis of RF I in this experiment is simply due to asynchrony in the culture. The small amount of late RF I synthesis, taken together with the evidence that the amount of RF I does not decrease significantly after phage DNA synthesis begins (Young & Sinsheimer, 1967*b*), indicates that it is not a major precursor to phage DNA. Pulse–chase experiments have qualitatively supported this conclusion, although the interpretation of these experiments has been difficult since some radioactivity was incorporated during the chase.

The kinetics of synthesis of RF II and X—the heterogeneous material labeled in a late pulse—suggest their turnover during the period of viral DNA accumulation. How they participate in the replication of viral DNA, whether as precursors or in some other capacity, remains to be elucidated.

Lindqvist & Sinsheimer (manuscript in preparation) have also implicated φX RF II in the synthesis of φX RF I and viral single-stranded φX DNA.

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